PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07H 21/00, 21/04, A61K 48/00

A1

(11) International Publication Number: **WO 98/52962**

(43) International Publication Date: 26 November 1998 (26.11.98)

(21) International Application Number: PCT/US98/10459

(22) International Filing Date: 19 May 1998 (19.05.98)

(30) Priority Data:
60/047,121 19 May 1997 (19.05.97) US

(71) Applicant *(for all designated States except US)*: MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: CAULFIELD, Michael, J. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

(74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: OLIGONUCLEOTIDE ADJUVANT

(57) Abstract

The invention provides oligonucleotide adjuvants useful for the generation of both a cell mediated (cytotoxic T lymphocyte) response and an antibody response to an antigen presented by the administration of a vaccine. Vaccine compositions including the adjuvant and methods of vaccination using the adjuvants are also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TITLE OF THE INVENTION
OLIGONUCLEOTIDE ADJUVANT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is entitiled to the benefit of U.S. Provisional Application 60/047,121, filed May 19, 1997.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable

REFERENCE TO MICROFICHE APPENDIX

Not applicable

FIELD OF THE INVENTION

This invention relates to the field of adjuvants and their use in vaccination.

BACKGROUND OF THE INVENTION

Vaccines are widely used to stimulate animal to mount an immune response against the antigen(s) in the vaccine. The immune response includes an immune memory component that helps to protect the animal against future exposure to infectious agents that present an antigen in the vaccine. The immune response can also include a cell-mediated response component.

Adjuvants are compounds useful in vaccine compositions. An adjuvant will cause an animal to mount a more vigorous immune response. Adjuvants can also cause an animal to develop a stronger immune memory for the antigen presented by administration of a vaccine.

Many compounds are useful as adjuvants in vaccine compositions. Since at least 1925 aluminum containing compounds have been used as adjuvants. Currently, the only adjuvants approved for use in human are aluminum salts, *e.g.*, aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate. More than 100 compounds or

formulations exhibiting some degree of adjuvant properties have been described (*See e.g.*,Vogel, F.K. and M.F. Powell (1995) A compendium of vaccine adjuvants and excipients, In: Vaccine Design, M.F. Powell and M.J. Newman, Eds., Pharm-Biotechnol. 6:141-228. All literature, patents and other publications cited herein are incorporated herein by reference in the entirety as background material).

Bacterial DNA has been reported to have immunostimulatory properties including the ability to induce natural killer (NK) cell activity and the induction of cytokines such as interferon (IFN) α/β and IFN-γ (Yamamoto, S., *et al.*. 1988. *In vitro* augmentation of natural killer cell activity of interferon α/β and -γ with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. Jpn. J. Cancer Res. (Gann) 79:866-873). Interferon- γ production was reported to be dependent on the induction of interleukin 12 (IL-12) and tumor necrosis factor-α (Halpern, M.D., *et al.*, 1996. Bacterial DNA induces murine interferon-γ production by stimulation of interleukin-12 and tumor necrosis factor- α. Cell. Immunol. 167:72-78). In contrast, mammalian DNA is reported to be non-mitogenic (Pisetsky, D.S. 1996. The immunologic properties of DNA. J. Immunol. 156:421-423).

Immunostimulatory bacterial DNA has been described as containing a palindromic sequence of at least six bases, *e.g.*, -GACGTC-, -AGCGCT-, or -AACGTT- (Yamamoto, S., *et al.*, 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity. J. Immunol. 148:4072-4076; Tokunaga, T., *et al.*, 1992. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. Microbiol. Immunol. 36:55-66). Similarly, Tokunaga, T. *et al.*, European Patent Application 0 468 520 A2, January 29, 1992, report that an immunostimulatory oligonucleotide must include a palindrome of at least 6 bases to be satisfactory (col. 3, lines 55-56; col. 11, lines 7-8; Table 7). Tokunaga *et al.*, also report that oligonucleotides of 10 bases or less have no immunopharmacological activity (col. 11, lines 34-37; Table 8).

The earlier reports on immunostimulating oligonucleotides addressed primarily non-specific or innate immunity, *e.g.*, NK cells have been shown to be activated *in vitro* and *in vivo* with oligonucleotides. More recently, it has been reported that oligonucleotides containing similar motifs are mitogenic for mouse B lymphocytes. These later reports did not observe a requirement for a palindromic sequence (Ballas, Z.K., *et al.*, 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol. 157:1840-1845) unless the oligonucleotide was 8 bases long (*See, e.g.*, Krieg, A. M., International Patent Application WO 96/02555, Feb. 1, 1996, col. 13, lines 36-38). That application also reported that an oligonucleotide must include an unmethylated CpG dinucleotide to stimulate the immune system of an animal (WO 96/02555).

The CpG motif is reported to be most stimulatory when it is presented within a larger oligonucleotide that includes a sequence of -GACGTT- or -GACGTC- (WO 96/02555, col. 6, lines 11-12). As to the size required for the oligonucleotide to be active, at least two reports state that testing indicated that oligonucleotides which include these sequences, and are less than 8 bases long, are non-stimulatory (*Id.* at col. 7, lines 19-20; Krieg, A.M. *et al.*, 1995. Nature 374:546-549, at col. 2, lines 14-18).

Krieg *et al.*, further reported that methylation of the cytosine base within the CpG motif resulted in a loss of activity. Thus, it is postulated that the lack of mitogenicity of mammalian DNA due to the fact that it is heavily methylated thereby inactivating CpG motifs.

Antibody secretion by a B cell line has been reported to increase with the addition of stimulatory CpG oligonucleotides (Krieg, A.M., *et al.*, 1995. Nature 374:546-549). Additionally, human B cells are reported to proliferate and produce polyclonal immunoglobulin in a T cell-independent manner upon culture with phosphorothioate oligonucleotides (Liang, H., *et al.*, 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. J. Clin. Invest. 98:1119-1129).

It is known that cytotoxic T lymphocytes (CTLs) kill virally- or bacterially-infected cells when their T cell receptors recognize foreign peptides associated with MHC class I and/or class II molecules. These peptides can be derived from endogenously synthesized foreign proteins, regardless of the protein's location or function within the pathogen. By recognition of epitopes from conserved proteins, CTLs may provide heterologous protection. In the case of intracellular pathogens, proteins secreted by or released from the pathogen are processed and presented by MHC class I and II molecules, thereby generating T-cell responses that may play a role in reducing or eliminating infection.

Most efforts to generate CTL responses have either used replicating vectors to produce the protein antigen within the cell [J.R. Bennink *et al.*, 311:578 (1984); J.R. Bennink and J.W. Yewdell, Curr. Top. Microbiol. Immunol. 163:153 (1990); C.K. Stover *et al.*, Nature 351:456 (1991); A. Aldovini and R.A. Young, Nature 351:479 (1991); R. Schafer *et al.*, J. Immunol. 149:53 (1992); C.S. Hahn *et al.*, Proc. Natl. Acad. Sci. (USA) 89:2679 (1992)], or they have focused upon the introduction of peptides into the cytosol [F.R. Carbone and M.J. Bevan, J. Exp. Med. 169:603 (1989); K. Deres *et al.*, Nature 342:561 (1989); H. Takahashi *et al.*, 344, 873 (1990); D.S. Collins *et al.*, J. Immunol. 148:3336 (1992); M.J. Newman *et al.*, 148:2357 (1992)].

The mechanism by which oligonucleotides containing CpG motifs induce immune activation is not fully known. Without desiring to be bound to a particular theory in the art, it has been reported that a key element is the induction of transcription of cytokine genes. In particular, rapid induction of high levels of IL-6 reportedly occurs both *in vitro* and *in vivo*, and transcription of IL-6 message is reported to be elevated in liver, spleen and thymus tissues upon administration of stimulatory oligonucleotides (Yi, A.-K., *et al.*, 1996. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. J. Immunol. 157:5394-5402). Finally, oligonucleotides containing CpG motifs have been reported to upregulate expression of MHC class II antigens (Krieg,

A.M., *et al.*, 1995. Nature 374:546-549). Taken together, these effects indicate that CpG oligonucleotides can augment antigen presentation thereby providing an adjuvant effect. Moreover, these adjuvants can provide a method to elicit a CTL response to an antigen presented in a vaccine.

However, while the oligonucleotide adjuvant of this invention can include a CpG dimer, the oligonucleotide does not satisfy the requirements of minimum lengths taught in the art. It is also found that a palindromic sequence is not required. Therefore, it is speculative whether the reported rationale for CpG oligonucleotide immunostimulatory activity applies to the oligonucleotides of the present invention.

SUMMARY OF THE INVENTION

It has now been discovered that an oligonucleotide consisting of six bases of the sequence 5' GACGTT 3,' 5' GAGCTT 3,' or 5'TCCGGA 3' are useful as a vaccine adjuvant in animals. These oligonucleotide adjuvants results in the generation of both a cell mediated response (cytotoxic T lymphocyte, CTL) and an antibody response to antigens presented by a vaccine.

As used herein, an adjuvant is a compound that improves the immune response of an animal to a vaccine antigen through one or more of the following mechanisms: (1) providing a depot of antigen for slow release of the antigen to the immune system, (2) causing enhanced uptake of the vaccine antigen by macrophages and other antigen presenting cells, (3) stimulation or activation of macrophages or neutrophiles (4) chemotactic recruitment of effector cells to the site of antigen injection, (5) enhanced proliferation of antigen-specific T and B cells, (6) increasing the precursor frequency of antigen-specific T and B cells, (7) increasing the amount and persistence of specific antibody, (8) increasing the breadth of the immune response in terms of antibody isotypes, (9) expanding the response to include multiple rather than single epitopes on the infectious agent, and (10) enhanced activation of memory T and B cells.

The oligonucleotide adjuvants of this invention, being a soluble molecule, would not be expected to provide a depot effect as described in pathway (1) above; however, its means of action could include any of the remaining 9 mechanisms either directly or indirectly.

As used herein, animal includes humans and non-human primates. Animal also includes domestic companion animals, *e.g.*, cats and dogs, and livestock, *e.g.*, chickens, cows, pigs, horses and sheep. Animal also includes all developmental stages of animals that are responsive to vaccination. In particular, pediatric administration is appropriate in animals and humans as soon as they are capable of responding to the antigen in a particular vaccine formulation.

Therefore, an aspect of this invention is a hexamer oligonucleotide adjuvant of the sequence 5' GACGTT 3,' 5' GAGCTT 3,' or 5'TCCGGA 3'. The oligonucleotide is useful in the administration of vaccine compositions to animals. In a preferred embodiment, the oligonucleotide has non-natural internucleotide linkages. The non-natural linkages can be only at the terminal linkages, the terminal and penultimate linkages or all the internucleotide linkages. In preferred embodiments, the internucleotide linkages are phosphorothioate linkages. In some embodiments, the oligonucleotide can have mixed linkages. In certain embodiments, adjuvant is encapsulated in a slow release delivery vehicle.

Another aspect of this invention is a vaccine formulation comprising one or more antigens and the oligonucleotide adjuvant. In embodiments of this aspect, the formulation can be liquid or lyophilized in dosage form. Many dosage forms are known in the art and can be applied herein. In embodiments of this aspect the oligonucleotide is present in the composition at a dose of from about 10 to about 10,000 µg per dose, from about 50 to about 5,000 µg per dose or from about 100 to about 500 µg per dose. In preferred embodiments the antigen is one or more antigens of hepatitis B, hepatocellular carcinoma antigens induced by hepatitis B virus, herpes simplex virus, human papilloma virus, hepatitis C virus envelope or core proteins, rotavirus bovine and human reassortants hepatitis A virus, human immunodeficiency virus

envelope, polymerase and core proteins, varicella, varicella zoster, *Streptococcus pneumonia* polysaccharide, *E. coli*, *Haemophilus influenza* polysaccharide, *Mycobacterium tuberculosis*, *Staphylococcus*, *Plasmodium* and *Schistosoma*. In certain most preferred embodiments, the antigen is hepatitis B surface antigen (HBsAg). In these embodiments the HBsAg protein antigen can be provided as a recombinant protein product prepared from yeast or an inactivated hepatitis B virus.

An aspect of this invention is a method of vaccination of an animal, including humans. The animal can be vaccinated prophylactically or therapeutically. The method of vaccination includes administering the oligonucleotide adjuvant of this invention and one or more antigens -- that is, the vaccine can be designed against one disease target or a combination of disease targets. Antigens that can be used in either or both therapeutic or prophylactic vaccines include antigens of hepatitis B, hepatocellular carcinoma induced by hepatitis B virus, herpes simplex virus, human papilloma virus, hepatitis C virus envelope or core proteins, rotavirus bovine and human reassortants hepatitis A virus, human immunodeficiency virus envelope, polymerase and core proteins, varicella, varicella zoster, *Streptococcus pneumonia* polysaccharide, *E. coli*, *Haemophilus influenza* polysaccharide, *Mycobacterium tuberculosis*, *Staphylococcus*, *Plasmodium* and *Schistosoma*.

In embodiments of this aspect the oligonucleotide and the antigen are administered contemporaneously. In other embodiments, they are administered simultaneously. In additional embodiments, the oligonucleotide and antigen are administered by intramuscular injection at the same site. In preferred embodiments of this aspect, the animal is a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Not applicable.

DETAILED DESCRIPTION OF THE INVENTION

It is now discovered that an oligonucleotide of only six bases having the sequence 5' GACGTT 3,' 5' GAGCTT 3,' or 5'TCCGGA 3' is an adjuvant useful in the vaccination of animals.

Vaccine compositions useful with the adjuvant of this invention can present antigens directly, *e.g.*, in the form of a particular protein(s) or peptide(s). Alternatively, vaccines can present an antigen(s) as a part of a complex biological or biochemical conglomerate, *e.g.*, as complete virus(es), virus-like particle(s), bacterial cell(s) or conjugate(s) including polysaccharide-protein conjugate molecules. These components can be combined to make a vaccine with multiple antigens.

A vaccine composition including at least one antigen is formulated to include the oligonucleotide adjuvant of this invention. For example, the antigen can be hepatitis B surface antigen as either a protein, a virus-like particle of recombinant protein produced in yeast (HBsAg), mammalian or insect cell culture or a peptide antigen derived from any of these.

The oligonucleotide adjuvant can be administered simultaneously or contemporaneously with the administration of the vaccine antigen. By simultaneously, it is meant that the antigen and adjuvant are administered together in the same formulation. By contemporaneously, it is meant that the antigen and the adjuvant are administered closely in time, *e.g.*, the adjuvant is administered within from about one minute to within about one day before or after the antigen is administered. Any contemporaneous time is useful. However, it will often be the case that when not administered simultaneously, the antigen and adjuvant will be administered within about one minute to within about eight hours, and preferably within less than about one to about four hours. When administered contemporaneously, the oligonucleotide adjuvant and antigen are administered at the same site on the animal. As used herein, the same site includes the exact location, but can be within about 0.5 to about 15 centimeters, preferably from within about 0.5 to about 5 centimeters.

The oligonucleotide can have various internucleotide linkages. It is preferred that at least the terminal internucleotide linkages be non-natural linkages. However, the terminal and penultimate linkages, or all the linkages can be non-natural. It is particularly preferred that oligonucleotide include phosphorothioate internucleotide linkages and it is most preferred that all of the internucleotide linkages are phosphorothioate. As used herein, an "S-ODN," is an oligodeoxynucleotide wherein all the internucleotide linkages are phosphothioate linkages. The natural phosphodiester linkage can also be appropriate in some cases..

Non-natural linkages are well known in the art and include methylphosphonates, phosphorothioates, phosphorodithionates, phosphoroamidites and phosphate ester linkages. Dephospho-linkages are also known, as bridges between nucleotides, and include siloxane, carbonate, carboxymethyl ester, acetamidate, carbamate, and thioether bridges. "Plastic DNA," having, for example, N-vinyl, methacryloxytethyl, methacrylamide or ethyleneimine internucleotide linkages. "Peptide Nucleic Acid" (PNA) is also useful and resists degradation by nucleases. Non-natural linkages can be mixed in the oligonucleotide. One need only test whether any particular linkage or combination of linkages in an oligonucleotide is appropriate for an adjuvant by using the assays described in the examples below

The purity of the oligonucleotide is very important for obtaining adjuvant activity. Residual chemicals, solvents, etc., from the process of synthesis should be removed by methods known in the art including chromatography on SEPHADEX™, reversed phase chromatography, high performance liquid chromatography, precipitation, dialysis, gel electrophoresis, or a combination thereof. Care should also be taken to ensure proper deprotection, which should be as complete as possible. Nevertheless, even with these precautions in mind, some syntheses will produce oligonucleotide that lack appropriate adjuvant activity. Therefore, it is most preferred that one test the oligonucleotide produced from each synthesis to confirm and assess the adjuvant activity. This lot to lot analysis will ensure that each lot will meet the

required minumum of adjuvant activity for the intended vaccine application. Lots of oligonucleotides that do not perform well in the assays can be discarded. It is demonstrated in the Examples that some lots do not act as adjuvants. One lot of the S-ODN 5' TCCGGA 3' did not show activity in a cell proliferation assay and is used as a control oligonucleotide in the Examples.

A further refinement to a formulation is to incorporate the oligonucleotide adjuvant into a delivery vehicle to provide for delayed release of the adjuvant over time. This can be accomplished by various means known in the art. The oligonucleotide can be formulated separately or together with at least one vaccine antigen. Examples of means of slow release delivery vehicles are encapsulation of the oligonucleotide adjuvant into PLGA microspheres (Deasy, P. B. et al. 1989. Preparation and characterization of lactic/glycolic acid polymers and copolymers. J. Microencapsul., 6: 369-378) liposomes, or bound (covalently or not) to carrier molecules such as proteins, polysaccharides or lipids.

As demonstrated below, immunization of an animal with the mixture of this adjuvant and HBsAg resulted in an increase in anti-HBsAg antibody titers after a single dose and the generation of HBs-specific CTLs after 3 doses. Therefore, the hexamer oligodeoxynucleotide adjuvant elicits the generation of both a cell mediated (cytotoxic T lymphocyte, CTL) response and an antibody response to an antigen presented by a vaccine.

A vaccine containing an antigen and the hexamer oligodeoxynucleotide adjuvant can be formulated and used as a prophylactic or therapeutic vaccine for a variety of disease targets including sexually transmitted diseases. A prophylactic vaccine is defined herein as a vaccine designed to elicit complete or partial protection from a disease caused by an infectious agent through the induction of specific immunity (*e.g.* antibodies specific for the infectious agent or its products, cytotoxic T lymphocytes (CTLs) specific for appropriately presented derivatives, *e.g.* peptides, associated with or produced by the infectious agent, or the activation of effector

cells, *e.g.* activated macrophages, mediated by cytokine production occurring as a result of T cell recognition of peptides derived from the infectious agent). In most cases, prophylactic vaccines are effective due to the activation of neutralizing antibodies specific for the infectious agent.

A therapeutic vaccine is defined as a vaccine designed to induce complete or partial remission of a chronic illness associated with an infectious agent. This vaccine activates cell-mediated immunity *(e.g.* specific CTLs) or the activation of effector cells (*e.g.* activated macrophages) mediated by cytokine production occurring as a result of T cell recognition of peptides derived from the infectious agent. The CTLs and activated macrophages lyse cells containing the infectious agent thereby eliminating the reservoir of infection. It is advantageous for a therapeutic vaccine to also induce specific antibodies to neutralize any infectious agents released from infected cells following lysis thereby preventing reinfection of host cells and tissues.

Vaccine antigens include antigens derived from hepatitis B (*e.g.*, S and preS-containing envelope proteins and core proteins); hepatocellular carcinoma antigens induced by hepatitis B virus, herpes simplex, human papilloma virus, hepatitis C virus (hepatitis C envelope or core proteins); rotavirus (including bovine and human reassortants); hepatitis A virus (*e.g.*, VAQTA®); human immunodeficiency virus ((HIV) HIV envelope proteins, HIV core proteins, HIV polymerase proteins); varicella or varicella zoster; antigens derived from bacteria including purified proteins, non-protein antigens, or mixtures of protein and non-protein antigens, *e.g.*, vaccines against *Streptococcus pneumonia,* (pneumococcal polysaccharide vaccines, pneumococcal polysaccharide-protein conjugate vaccines), *E. coli, Haemophilus influenza* (including polysaccharide-protein conjugate vaccines), *Mycobacterium tuberculosis* vaccines and vaccines against bacteremia caused by *Staphylococcus* (*e.g., Staphylococcus aureus*) and other bacteria; antigens derived from parasites that cause diseases such as malaria (*Plasmodium falciparium*) and schistosomiasis (*Schistosoma mansoni*); and antigenic material derived from human or animal

tumors. The above vaccines can be directed against various serotypes of bacteria and viruses as appropriate.

A vaccine including the adjuvant oligonucleotide of this invention can be formulated as appropriate for the subject animal. Formulations can be liquid or lyophilized. Many vaccine formulations are known in the art and can be used by substituting the adjuvant of this invention for the adjuvant previously known in the art. An example of a liquid formulation vaccine is one for hepatitis B. The formulation is an injectable solution containing from about 5 to about 40 µg, preferably from about 5 to about 20 or from about 5 to about 10 µg, of hepatitis B surface antigen (prepared, *e.g.*, as described in U.S.Patent Nos. 4,769,238 9/6/88; 4,935,235 6/19/90; and 5,196,194 3/23/93) in a formulation with a pharmaceutically acceptable buffer (*e.g.*, phosphate) at a pH of about 5.5 to about 8.0. The formulation contains from about 10 to about 10,000 µg of the 5' GACGTT 3' oligonucleotide adjuvant, preferably from about 50 to about 5,000 µg and most preferably from about 100 to about 1,000 µg. Particular formulations may require particular amounts within these ranges, for example, about 200, 500, 750, 1,500, 2,500 or 3,500 µg, or other amounts not listed here, of the adjuvant oligonulceotide can be used.

An injectable formulation can be prepared by mixing a double-strength liquid formulation of antigen with a double strength liquid (or previously frozen) solution of an oligonucleotide adjuvant such as 5' GACGTT 3'. Alternatively, a vaccine can be prepared by mixing from about 10 to about 1,000-2,000 µg of the oligonucleotide 5' GACGTT 3' in a pharmaceutically acceptable buffer with antigen formulated with aluminum hydroxyphosphate or another adjuvant (such as aluminum hydroxide, aluminum phosphate, calcium phosphate, saponins, non-ionic block copolymers, oil in water emulsions, or cytokines) and/or with excipients. Also, a vaccine can be formulated to contain from about 5 to about 20 µg of HBsAg, from about 10 to about 1,000-2,000 µg of the oligonucleotide 5' GACGTT 3', and from about 0.1 to about 100 µg of a cytokine such as interleukin 12.

An example of a lyophilized formulation is a lyophilized live, attenuated virus such as VARIVAX® (a vaccine for varicella or chicken pox) with a buffered solution containing from about 10 to about 1,000-2,000 µg of the oligonucleotide 5' GACGTT 3'.

EXAMPLE 1

Materials and Methods

The following methods are used generally throughout the examples.

Oligonucleotide (ODN) Synthesis

ODNs having phosphodiester or phosphorothioate internucleotide linkages were synthesized at Midland Certified Reagent Co., Midland TX. ODNs with phosphodiester backbones were synthesized using the O-cyanoethyl phosphoroamidite chemistry (Sinha, N.D., Biernat, J, and Koster, H. 1983. Tetrahedron Letters 24:5843-5846). ODNs with phosphorothioate backbones were synthesized as described in Iyer, R.P., Egan, W., Regan, J.B., Beaucage, S.L. 1990. 3H-1,2-benzodithiole-3-one 1,1-dioxide as an improved sulferizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. J. American Chem. Soc. 112: 1253-1254.

ODNs were purified by trityl selective purification (TSP) on a reverse phase column or by high pressure liquid chromatography (HPLC) (ZORBAX™ Bioseries Oligo (21.2 x 250 mm) anion exchange column).. In the TSP procedure, full-length ODNs containing a terminal trityl group are selectively bound to the reverse phase column. After washing, the trityl is removed and the ODN eluted from the column in ammonium acetate, acetonitrile, and trifluoroacetate and then dried to remove the volatile solvents. In the HPLC procedure, a buffer containing sodium sulfate and urea was used to purify the ODN.

For either procedure, a desalting step on a SEPHADEX™ column aids in the removal of salts, *e.g.*, salts of sulfate, urea or amonium, from the final product and is strongly recommended. It may be possible to use SEPHADEX™ G-25 for desalting, but is not

preferred. The purification provided by a SEPHADEX™ G-10 desalting column is preferred.

While the purification methods noted above were used to prepare the ODNs used herein, one of ordinary skill in the art will recognize that there are other methods known and used in the art. Different methods can be used. However, it is apparent from the results presented herein that when one prepares synthetic ODNs one needs to purify the ODNs away from residual impurities. While desalting on SEPHADEX™ G-10 is preferred, one can use other appropriate methods.

All of the internucleotide linkages of the oligonucleotide used in these examples were phosphorothioate linkages (S-ODNs), except where indicated.

Anti-HBsAg EIA (total antibody)

A microtiter plate modification of the AUSAB EIA kit (Abbott Labs, N. Chicago, IL) was used to quantify antibodies to hepatitis B surface antigen (HBsAg). Costar EIA 96-well flat bottom plates (Costar, Cambridge MA, #3591) were coated overnight at 4°C with recombinant HBsAg (prepared *e.g.*, U.S.Patent Nos. 4,769,238 9/6/88; 4,935,235 6/19/90; and 5,196,194 3/23/93; all of which are incorporated herein by reference in their entirety) at 4 µg/ml in Tris-saline, pH 9.5. Plates were washed 3 times with PBS and then blocked with 175 µl/well of PBS/5% FCS/ 0.1% azide for 2 hrs at room temperature or overnight at 4°C. Five-fold serial dilutions were made (in duplicate) in 8 consecutive wells of the plate for each serum sample. The plates were then incubated overnight at 4°C. After 3 wash cycles with PBS (using a TiterTech plate washer [ICN, Huntsville, AL]), a developing reagent (Abbott AUSAB EIA kit) consisting of equal volumes of biotin-conjugated HBsAg and an anti-biotin-enzyme conjugate was added to each well of the plate. After 4 hrs at room temperature, the plates were washed 6 times and then 100 µl per well of OPD substrate (Abbott) was added to each well. The reaction was stopped after 30 minutes with the addition of 50 µl per well of 1 N

H_2SO_4. Optical densities were read at 490 nm and 650 nm using a Molecular Devices microplate reader (Molecular Devices, Menlo Park, CA). Anti-HBsAg titers (in mIU/mL) were calculated by the Softmax computer program (version 2.32) using a standard curve generated using a 4-parameter fit algorithm. Since the assay is species-independent, a set of human serum standards (Abbott quantitation kit) was used to generate the standard curve so that titers could be quantified relative to a reference standard in mIU/mL.

Anti-HBsAg EIA (isotype-specific)

Microtiter plates were coated with HBsAg and blocked as described above. Five-fold serial dilutions were made (in duplicate) in 8 consecutive wells of the plate for each serum sample. The plates were then incubated overnight at 4°C. After 3 wash cycles with PBS (using a TiterTech plate washer), alkaline phosphatase-conjugated goat anti-mouse immunoglobulin reagents specific for mouse IgG1 or mouse IgG2a isotypes (Southern Biotechnology Associates, Birmingham, AL) were added at a final dilution of 1:2000. After 2 hr at 37°C, the plates were washed 6 times using a Titertech plate washer, and then 60 µl per well of the enzyme substrate (p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO) dissolved at 1 mg/mL in Tris saline, pH 9.5) was added. After 30 minutes at room temperature, the reaction was stopped with the addition of 60 µl/well of 3N NaOH. Optical densities were read at 405 nm using a Molecular Devices microplate reader. Data were collected using the Softmax computer program (version 2.32) (Molecular Devices, Menlo Park, CA). A standard curve was generated using mouse monoclonal anti-HBsAg antibodies of the IgG1 (catalogue # 16021, Pharmingen, San Diego, CA) or IgG2a (cat. # 16011D, Pharmingen) isotypes.

Antibody concentrations relative to each isotype standard were calculated as described previously (Caulfield, M.J., and D. Shaffer. 1984. A computer program for the evaluation of ELISA data obtained using an automated microtiter plate absorbance reader. *J. Immunol. Methods* 74:205-215). Briefly, to calculate titers, an OD

value of 0.1 units was set as the endpoint. The log 5 titer (t) is determined by interpolation using the following formula:

$$t = x - ((0.1 - L)/(H - L)$$

where L = OD value of the first log 5 dilution giving an OD value below 0.1; H = OD value of the log 5 dilution closest to, but above the cutoff (0.1); x = the well number that has the OD value L.

The antibody concentration (c) in experimental samples is determined by comparing the endpoint titer in experimental wells with that of the standard curve by the following formula:

$$c = A \times 5^{(t - s)}$$

where A = the antibody concentration of the standard; s = the log 5 titer of the standard; t = the log 5 titer of the unknown. For example, if the log 5 endpoint titer of the standard (100 ng/ml) is 2.6 and the value of the unknown is 3.4, the concentration of antibody in the unknown would be:

$$c = 100 \times 5^{(3.4 - 2.6)} = 362 \text{ ng/ml}$$

Proliferation assay

Mouse spleen cells were cultured for ~48 hours in round bottom 96-well culture plates (Costar #3799) at 2 x 10^5 cells per well in 200 µl "K" medium. ["K" medium consists of RPMI-1640 medium (Gibco-BRL, Grand Island, NY, Cat. # 11875-093) supplemented with 10% heat-treated (56°C, 30 minutes) fetal bovine serum (Gibco BRL #16000-044), antibiotic mixture (Gibo-BRL # 15140-122) containing 100 U/mL penicillin and 100 µg/mL streptomycin, 10 mM HEPES buffer Gibco-BRL #15630-080, 2 mM L-glutamine (Gibco-BRL #25030-081), and 5 x 10^5 M β-mercaptoethanol (Sigma #M-7522). The cells were cultured with various concentrations of oligonucleotides (Midland Certified Reagent Co., Midland TX) or with

lipopolysaccharide (*E. coli* LPS O111:B4, Sigma #L-3012) as a positive control. On day 2, 0.1 mL of culture supernatant was removed from each well and replaced with 0.1 mL of culture medium containing 10 μCi/mL of ^{3}H-thymidine (Amersham, Arlington, IL, cat. # TRK 7637). Four hours later, the contents of each well were harvested onto glass fiber mats (Wallac, Turku, Finland, cat. #1205-404) using a TomTec MachII harvester (Wallac, serial # 145). The mats were dried and then transferred to plastic bags (Wallac #1205-411) which were sealed after the addition of scintillation fluid. Radioactive thymidine incorporation was quantified using a BetaPlate 1205 scintillation counter (Wallac, Turku, Finland). The counts per minute (cpm) in experimental and control wells were compared to determine a stimulation index (cpm experimental/cpm control).

Cytotoxic T Lymphocyte Assays (CTL assays)

The CTL assays were performed as reported in Ulmer, J.B., *et al.* 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259: 5102:1745-9. Briefly, BALB/c mice were injected three times with a vaccine formulation consisting of HBsAg and the 5' GACGTT 3' oligonucleotide. A single cell suspension of effector cells was then prepared and cultured *in vitro* with HBsAg peptide (28-39)-pulsed syngeneic stimulator cells. The cell suspension was assayed 7 days later for CTL activity against ^{51}Cr-labeled P815 cells.

The syngeneic stimulator cells were prepared as a single cell suspension from the spleens of unimmunized BALB/c mice as follows. After lysis of red blood cells with ammonium chloride buffer (Gibco BRL ACK buffer), the cells were washed by centrifugation for 10 minutes at 1200 rpm (Jouan centrifuge model CR422), resuspended in DMEM culture medium (Gibco BRL #11965-092), and then irradiated using a ^{60}Co source to deliver 2,000 - 4,000 rads. The cells were then pulsed with a 10 μM final concentration of the H-2 K^{d} peptide HBsAg (28-39) (Chiron Mimetopes, Clayton, Victoria, Australia) which has the sequence IPQSLDSWWTSL (SEQ ID NO:1) (Schirmbeck, R., *et*

al., 1994. Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implication for the pathogenesis of HBV-induced hepatitis. *J. Virol.* 68:1418-1425). The cells were mixed approximately every 20 minutes for 1.5 - 2.5 hrs and then washed 3 times with RPMI-1640 medium. Effector cells were prepared as single cell suspensions from spleens of immunized mice as described and then co-cultured with an approximately equal number of peptide-pulsed stimulator cells for 7 days at 37° C (5% CO_2) in "K" medium.

P815 ($H\text{-}2^d$) mouse mastocytoma cells (ATCC, Rockville, MD) were radiolabeled by overnight culture with 0.5 - 1.2 mCi ^{51}Cr (Amersham, cat. # CJS.4) added to 75 cm^2 culture flasks (Costar #3376) containing ~ 5 x 10^5 cells per mL in a volume of 10 mL. The labeled cells were centrifuged at 1200 rpm for 5 minutes and the supernatant removed by aspiration. The cells were washed, counted, resuspended in DMEM culture medium at ~10^6 cells per mL and then pulsed with 10 µM HBsAg (28-39) peptide at 37° C for 2-3 hr with frequent mixing. The target cells were then washed and adjusted to 10^5 cells per mL for plating.

Meanwhile, effector cells from the 7 day restimulation cultures were harvested, washed, and added to triplicate wells of V bottom microtiter plates (Costar #3898) at 60 x 10^5, 30 x 10^5, 15 x 10^5, and 7.5 x 10^5 cells per mL. The ^{51}Cr-labeled target cells were plated at 10^4 cells per well in 100 µl "K" medium to achieve effector:target ratios of 60:1, 30:1, 15:1, and 7.5:1. Triplicate wells containing only target cells cultured in 0.2 mL of medium served as controls for spontaneous ^{51}Cr release whereas triplicate wells containing target cells cultured in 0.2 mL of medium containing 1.0 % Triton X-100 detergent (Sigma #T6878) served as controls for maximum ^{51}Cr release. The plates were incubated for 4 hours at 37°C in a 5% CO_2 incubator and then centrifuged at 1200 rpm for 5 minutes to pellet the remaining target cells. The supernatants (20 µl) were then harvested using an Impact multichannel pipetor (Matrix Technology, Lowell MA, model #6622) and then transferred to Betaplate filter mats (Wallac #1205-402). The mats were dried and then transferred to plastic bags which were sealed

after the addition of ~11 mL of scintillation fluid. A Betaplate model 1205 scintillation counter (Wallac) was used to quantify the radioactive ^{51}Cr contained in each spot on the mat corresponding to each well of the original 96-well plate. The % specific lysis = 100 x [(experimental cpm - spontaneous cpm) / (maximum release cpm - spontaneous cpm)].

EXAMPLE 2

Selection of oligonucleotide sequences

WO 96/02555 teaches that to be immunostimulatory, oligonucleotides require sequences containing CpG motifs and must be at least 8 nucleotides long (WO 96/02555, col. 13, lines 19-20, *see also*, Krieg, A.M., *et al.*, 1995. Nature 374:546-549, col. 2, lines 14-18). Further, if the oligonucleotides were 8 nucleotides long they also require a palindromic sequence of at least 6 nucleotides (WO 96/02555, col. 13, lines 36-38). EP 0 468 520 teaches that to be imunostimulatory, oligonucleotides require a palindromic sequence of at least 6 nucleotides long to be satisfactory and must be at least 10 nucleotides long overall to be active (EP 0 468 520, col. 11, lines 34-37 & Table 7).

Therefore, S-ODNs less than 8 nucleotides in length were tested for the ability to induce proliferation of mouse spleen cells *in vitro*. It has been found that a nonpalidromic hexamer oligonucleotide consisting of the sequence 5' GACGTT 3' (lot # 050796-114) induced proliferation of mouse spleen cells whereas a particular lot of palindromic CpG oligonucleotide consisting of the sequence 5' TCCGGA 3' (lot # 050796-115) had no activity in this experiment and was used as a control thereafter. Proliferation was measured by the incorporation into DNA of ^{3}H-thymidine as described above. The amount of proliferation induced with the 5' GACGTT 3' oligonucleotide was dose-dependent. The 100 µM dose resulting in a stimulation index (SI) of > 10-fold. The two oligonucleotide lots described in Table 1 were also used in examples 2 - 6.

Table 1. Proliferation of DBA/2 mouse spleen cells in response

to the 5' GACGTT 3' oligonucleotide

[μM oligo]	5' GACGTT 3' (lot # 050796-114)		5' TCCGGA 3' (lot # 050796-115	
	cpm	SI	cpm	SI
300	2,106	7.73	264	0.97
100	3,206	11.76	529	1.94
30	408	1.50	455	1.67
10	196	0.72	355	1.30
3	229	0.84	420	1.54
1	337	1.24	290	1.06
0.3	304	1.12	305	1.12

EXAMPLE 3

Adjuvant effect of the 5' GACGTT 3' oligonucleotide in elevating antibody titers in mice.

The ODNs of this invention are adjuvants that elevate antibody titers raised against an antigen. The formulation used in this example is the simple addition of a source of HBsAg with an oligonucleotide hexamer consisting of the sequence 5' GACGTT 3'. All of the internucleotide linkages of the oligonucleotide used in these examples were phosphorothioate linkages (S-ODNs), except where indicated. However, alternative linkages can also be used. The efficacy of an oligonucleotide adjuvant candidate can be very simply tested following the method of this example. The results can be compared to the results presented here for the 5' GACGTT 3' S-ODN. Likewise, other antigens can be formulated and tested following this example. Finally, vaccine formulations including an adjuvant in addition to 5'GACGTT3', *e.g.*, an aluminum salt adjuvant, can be designed, tested for efficacy and compared to formulations containing only one of the adjuvants.

In this example, the HBsAg antigen was the Recombivax HB® final aqueous product (FAP) -- that is, Recombivax HB® without the aluminum adjuvant. This antigen was used in formulations with the 5' GACGTT 3' adjuvant. However, other sources of hepatitis B surface antigen are known in the art and are also appropriate for formulation with 5'GACGTT 3', *e.g.*, the hepatitis B surface antigen in ENERGIX B® (SmithKline Beecham, Inc., King of Prussia, PA). Also, hepatitis B surface antigen prepared from mammalian cells, *e.g.*, Chinese hamster ovary (CHO) cells, or insect cells, would also be appropriate.

The mixture of HBsAg plus 5' GACGTT 3' was used to immunize BALB/c mice. The mice were bled at intervals to obtain serum that was tested for anti-HBsAg antibodies. A separate set of mice were immunized with HBsAg plus the control palindromic CpG oligonucleotide 5' TCCGGA 3.' The dose of HBsAg was 1 µg and the dose of the oligonulceotides was approximately 0.3, 3.0 or 30 µg per injection.

As shown in Table 2, a single injection of the mixture of the 5' GACGTT 3' with HBsAg on day 0 resulted in approximately a 40-fold increase in the geometric mean titer (GMT) of the anti-HBsAg antibody response of mice when compared with mice injected with the HBsAg only. Formulation with the 5' TCCGGA 3' S-ODN (control lot) had little or no adjuvant effect for HBsAg.

Table 2. Post-dose 1 anti-HBsAg response to HBsAg protein ± S-ODN hexamer

Immunogen	*Adjuvant*	*[oligo]*	*Anti-HBsAg GMT (mIU/mL)*
HBsAg (1 µg)	$Al(OH)PO_4$		750.5
	none		3.6
	5' GACGTT 3'	30 µg	186.3
	lot # 050796-114	3 µg	11.4
		0.3 µg	3.4
	5' TCCGGA 3'	30 µg	24.0
	lot# 050596-115	3 µg	6.0
		0.3 µg	7.0

n = 10 BALB/c mice per group
i.m. injection day 0; assay d. 42

As shown in Table 3, two injections of the formulation of 5' GACGTT 3' + HBsAg on day 0 and day 42 resulted in day 84 anti-HBsAg titers as much as 100-fold higher than that attained without the 5' GACGTT 3' oligonucleotide adjuvant. The titer achieved using about 30 µg of 5' GACGTT 3' was equivalent to that induced with HBsAg formulated with Merck aluminum adjuvant. Formulation with the 5' TCCGGA 3' (control) oligonucleotide showed a small adjuvant effect for HBsAg when formulated at higher amounts.

Table 3. Post-dose 2 anti-HBsAg response to HBsAg protein ± hexamer

Immunogen	*Adjuvant*	*[oligo]*	*Anti-HBsAg GMT (mIU/mL)*
HBsAg (1 µg)	**$Al(OH)PO_4$**		98,129
	none		248
	5' GACGTT 3'	30 µg	102,592
	lot# 050596-114	3 µg	2,115
		0.3 µg	103
	5' TCCGGA 3'	30 µg	961
	lot# 050596-115	3 µg	621
		0.3 µg	69

n = 10 BALB/c mice per group
i.m. injection day 0, 42; assay d. 84

EXAMPLE 4

Effect of 5' GACGTT 3' S-ODN on antibody isotype.

The isotype profile of an immune response is dependent on the activation of a population of helper T (TH) cells of the TH1 type or of the TH2 type. These two populations of TH cells exhibit different functions as a result of the production of distinct cytokines. TH1 cells produce IL-2 and interferon-γ (IFN- γ) whereas TH2 cells produce IL-4 and IL-10 (Mosmann, T.R, *et al.*, 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348; Cher, D.J., and T.R. Mosmann. 1987. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. *J. Immunol.* 138: 3688-3644). Responses dominated by antibodies of the IgG1 isotype are characteristic of a TH2 response whereas responses consisting of IgG2a isotype antibodies reflect the induction of a TH1 type response (Coffman, R.L., *et al.*, 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 102:5).

Table 4 contains an analysis of the isotype distribution of the murine antibody response to HBsAg formulated with an aluminum adjuvant compared with the oligonucleotide adjuvant. The results show that immunization with HBsAg plus the 30 µg dose of 5' GACGTT 3' induced a dramatic increase in both IgG1 and IgG2a isotype responses when compared with the response to HBsAg alone. Furthermore, the 5' GACGTT 3' oligonucleotide induced as much as a five-fold increase in anti-HBsAg antibodies of the IgG2a isotype relative to the group receiving HBsAg plus the aluminum hydroxyphosphate adjuvant. The elevated IgG2a isotype response was associated with a decrease in the ratio of IgG1 to IgG2a anti-HBsAg antibodies. These results show the induction of a TH1 response in addition to the TH2 response and demonstrate that the 5' GACGTT 3' oligonucleotide induces broader immune activation than does an aluminum adjuvant. The control lot of the 5'TCCGGA3' S-ODN produced a small increase in IgG1 only.

Table 4. Isotype profile of the anti-HBsAg response to HBsAg protein ± S-ODN hexamers

		Anti-HBsAg antibody isotype	
HBsAg (1μg) +	*[oligo]*	*IgG1 (μg/mL)*	*IgG2a (μg/mL)*
Al(OH)PO_4		1281	4.2
none		2.6	0.02
5' GACGTT 3'	30 μg	764.3	20.0
lot# 050596-114	3 μg	52.7	12.7
	0.3 μg	29.3	0.03
5' TCCGGA 3'	30 μg	100.8	0.5
lot# 050596-115	3 μg	35.2	0.16
	0.3 μg	7	0.06

n = 10 BALB/c mice per group
i.m. injection day 0, 42; assay d. 84

EXAMPLE 5

Adjuvant effect of 5' GACGTT 3' S-ODN for low responder mice.

A significant proportion of humans are non-responders to a standard 3-dose regimen of the current hepatitis B vaccines (Alper, C.A., *et al.*, 1989. Genetic prediction of nonresponse to hepatitis B vaccine. *J. Eng. J. Med.* 321:708-712). This problem was addressed using the 5' GACGTT 3' adjuvant in a preclinical animal model. Low responder C3H mice were immunized with two doses of 5' GACGTT 3' + HBsAg.

As shown in Table 5, mice that received the ~30 μg dose of the 5' GACGTT 3' (plus HBsAg) co-injected into the same i.m. site developed a good anti-HBsAg titer that was 10-fold higher than the response to HBsAg alone. Injection of the oligonucleotide in the

opposite leg from the antigen did not result in an adjuvant effect suggesting that the 5' GACGTT 3' does not simply increase innate immunity; rather, the oligonucleotide elicits a localized adjuvant effect at the site of injection or, possibly, in the draining lymph node. In this experiment, the control lot of S-ODN 5'TCCGGA3' did not produce a significant response.

Table 5. Low responder C3H mouse response to HBsAg ± S-ODN hexamer (post-dose 2)

		Anti-HBsAg GMT (mIU/mL)	
HBsAg (1 µg) +	*[oligo]*	co-injected	contralateral injections
$Al(OH)PO_4$		588	
none		18	
5' GACGTT 3'	30 µg	208	13
lot# 050596-114	3 µg	14	11
	0.3 µg	10	7
5' TCCGGA 3'	30 µg	37	9
lot# 050596-115	3 µg	30	7
	0.3 µg	9	10

n = 10 C3H mice per group
i.m. injection day 0, 42; assay d. 63

EXAMPLE 6

5' GACGTT 3' S-ODN enhances the specific cytotoxic T lymphocyte (CTL) response to an antigen.

After three injections of the HBsAg plus 5' GACGTT 3' S-ODN, spleen cells from BALB/c mice were restimulated *in vitro* with HBsAg peptide (28-39) and then assayed 7 days later for CTL activity against ^{51}Cr-labeled P815 cells. As shown in Table 6, the 5' GACGTT 3' oligonucleotide was a potent adjuvant for the induction of HBs-specific CTLs. There was no lysis of control P815 cells not pulsed with the HBsAg peptide indicating that lysis of the HBsAg peptide-pulsed

cells was the result of activation of specific CTLs rather than natural killer (NK) cells that would be expected to lyse target cells indiscriminately.

Without wishing to be bound by any particular theory, it is noted that the adjuvant effect of the 5' GACGTT 3' S-ODN may have a sequence specific component since injection of mice with HBsAg + a control hexamer consisting of the sequence 5' TCCGGA 3' did not result in significant activation of specific CTLs in this experiment. However, different lots of S-ODNs containing this sequence were found to have adjuvant activity in separate experiments (see example 10). HBsAg formulated with an aluminum adjuvant (Recombivax HB®) or with no adjuvant did not induce an HBs-specific CTL response.

Table 6. Induction of specific CTLs from mice immunized with the 5' GACGTT 3' adjuvant

Immunogen (1.0 µg)	Adjuvant	E:T ratio	*% Specific lysis* Target 1 no peptide	Target 2 HBsAg (28-39)
	5' GACGTT 3'			
HBsAg	(30 µg)	60	-14	68
	lot# 050596-114	30	-14	53
		15	-12	33
		7.5	-11	20
HBsAg	5' TCCGGA 3'	60	-5	6
	(30 µg)	30	-15	4
	lot# 050596-115	15	-10	3
		7.5	-6	0
HBsAg	none	60	-19	-2
		30	-20	-5
		15	-17	-2
		7.5	-9	-1
HBsAg	$Al(OH)PO_4$	60	-16	-11
		30	-16	-7
		15	-18	-7
		7.5	-18	-7

Mouse strain: BALB/c

Injection schedule: d. 0, d. 42, d. 168

CTL Response: effector cells were restimulated (d. 181) with HBs 28-39 peptide pulsed spleen cells for 7d.

Target 1: Max lysis=2757.3 CPM, spontaneous lysis=1084.7 CPM

Target 2: Max lysis=3087.2 CPM, spontaneous lysis=862.5 CPM

%Specific lysis = 100 x [(experimental cpm - spontaneous cpm) / (maximum release cpm - spontaneous)]

In contrast to the results shown in Table 6, it has been reported that soluble HBsAg can induce a specific CTL response in mice (Schirmbeck, R., *et al.*, 1994. Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implication for the pathogenesis of HBV-induced hepatitis. J. Virol. 68:1418-1425; Schirmbeck, R., *et al.*, 1994. Selective stimulation of murine cytotoxic T cell and antibody responses by particulate or monomeric hepatitis B virus surface (S) antigen. E. J. Immunol. 24:1088-1096; Schirmbeck, R., *et al.*, 1995. Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. J. Virol. 69:5929-5934). The procedures used in these reports differ from the present methods in the route of injection (i.p. or s.c. vs. i.m.) and *in vitro* restimulation conditions (5 -7 days post immunization vs. 13 days post-immunization) and in the addition of grow factors during *in vitro* restimulation (the Schirmbeck group report that a source of IL-2 to their CTL cultures whereas none was added herein). IL-2 amplifies the response through the expansion of CTL precursors and thereby exaggerating the effect of immunization. Schirmbeck *et al.* are in agreement with the present results showing that immunization with a conventional HBV vaccine (formulated with aluminum adjuvant) does not induce a CTL response (Schirmbeck, R., *et al.*, 1994.E. J. Immunol. 24:1088-1096). Furthermore, Schirmbeck et al. reported that conditions favoring the development of a CTL response (soluble antigen) resulted in a lower antibody response to the HBsAg. In direct contrast, using a hexamer S-ODN adjuvant of the present invention, 5' GACGTT 3', one is able to generate both a high antibody titers and HBs-specific CTLs.

EXAMPLE 7

Timing of 5' GACGTT 3' adjuvant injection relative to antigen injection

As shown in Table 7, the 5' GACGTT 3' S-ODN adjuvant is effective in eliciting an immune response to an antigen, in this case HBsAg, when contemporaneously injected into the same intramuscular site of an animal. In this experiment the adjuvant was injected one day before, the same day, or one day after injection of the antigen. In a control group, mice were injected with HBsAg without adjuvant. Contemporaneous injection resulted in a 4- to 14-fold enhancement of anti-HBsAg titers relative to the control group.

Table 7. Timing of 5' GACGTT 3' adjuvant injection relative to HBsAG antigen injection.

Immunogen (day 0)	Adjuvant (~ 30 µg)	Adjuvant regimen*	Anti-HBsAg GMT (mIU/mL)
HBsAg (1 µg)	none	none	258
	5' GACGTT 3'	day -1	1068
	5' GACGTT 3'	day 0 (+ 2 hrs)	1850
	5' GACGTT 3'	day +1	3618
	5' GACGTT 3'	day +3	323
	5' GACGTT 3'	day +5	336
	5' GACGTT 3'	day +7	80

n = 10 BALB/c mice per group
Injection site: intramuscular (T.A. muscle)
Antigen injection (i.m.) schedule: d. 0, d. 42
5' GACGTT 3' lot # 112796-81

*Timing of adjuvant injection (into the same i.m. site) relative to HBsAg injection

EXAMPLE 8

Proliferation of BALB/c mouse spleen cells cultured with ODNs

As noted above, in preferred embodiments, the internucleotide linkages are phosphorothioates. In this experiment, proliferation of spleen cells as induced by a number of lots of synthetic oligonucleotides was studied. The results shown in Table 8 indicate that ODNs containing phosphorothioate internucleotide linkages (S-ODNs) are stronger mitogens than ODNs containing conventional phosphodiester (O-ODNs) internucleotide linkages. It is also notable that S-ODN hexamers are immunostimulatory in contrast to the predictions of Kreig et al. (Krieg, *et al.*, 1995). In this experiment, the lot to lot variability of the adjuvant activity of synthetic oligonucleotides is clearly seen by comparison of lots #308, 309 & 360 of S-ODN 5'GACGTT 3' and lots #115 & 361 of S-ODN 5'TCCGGA3'.

Table 8. Proliferation of BALB/c mouse spleen cells cultured with ODNs

Sequence	lot #	Linkage	[ODN]	cpm	stimulation index
GACGTT	052997-360	S	100 µM	75,959	53.3
			30 µM	11380	8.0
			10 µM	3,559	2.5
			3 µM	1,952	1.4
	052897-308	S	100 µM	58,447	41.0
			30 µM	7,805	5.5
			10 µM	2,896	2.0
			3 µM	2,272	1.6
	052897-309	S	100 µM	44,133	30.9
			30 µM	10,615	7.4
			10 µM	3,708	2.6
			3 µM	2,564	1.8
	060497-116	O	100 µM	538	0.5
			30 µM	428	0.4
			10 µM	457	0.4
			3 µM	565	0.5
TCCGGA	0529-97-361	S	100 µM	15,845	11.1
			30 µM	11,095	7.8
			10 µM	6,768	4.7
			3 µM	3,824	2.7
	052997-367	O	100 µM	2,244	1.6
			30 µM	772	0.5
			10 µM	930	0.7
			3 µM	1,130	0.8
GAGCTT	052997-365	S	100 µM	12,759	8.9
			30 µM	7,023	4.9
			10 µM	4,445	3.1
			3 µM	1,387	1.0

The experiment was conducted as in Example 1 except that spleen cells from BALB/c mice were cultured in triplicate in 96-well plates for 2 days with the indicated ODNs. ^{3}H-thymidine was added during the last 4 hours of incubation to assess proliferation. Unstimulated cultures had a mean counts of 1274 cpm.

The reason for the lot to lot variability of the ODNs in their ability to induce a lymphoproliferative response is unknown, however, it may relate to trace contaminants of urea or ammonium ions that may have been incompletely removed by size exclusion chromatography of large scale ODN preparations on SEPHADEX™ columns.

EXAMPLE 9

Low responder C3H mouse responsd to S-ODN adjuvants

In the examples that follow, data are presented showing that the immunostimulatory activity of ODNs, as measured by lymphoproliferation, does not always correspond to adjuvant properties *in vivo*. Example 9 examines the sequence requirements for an adjuvant effect whereas Example 10 addresses the effect of the internucleotide linkage on adjuvanticity.

The results shown in Table 9 indicate that phosphorothiate ODNs lacking a CpG dinucleotide can have adjuvant properties for a protein vaccine. Groups of 10 C3H mice were co-injected i.m. on days 0 and 42 with HBsAg protein mixed with the indicated concentration of phosphorothioate ODNs containing a CpG or a GpC motif. Control mice were injected with HBsAg protein formulated with or without an aluminum adjuvant. Anti-HBsAg titers were determined on sera collected 3 weeks post-dose 2.

Table 9. Low responder C3H mouse response to HBsAg ± S-ODN

HBsAg (1 µg) +	[S-ODN]	Anti-HBsAg GMT (mIU/mL)
$Al(OH)PO_4$		30 (34)
none		5 (2)
5' GACGTT 3'	30 µg	122 (92)
(lot # 072397-354 S.I.=37.0)	3 µg	57 (64)
	0.3 µg	42 (33)
5' GAGCTT 3'	30 µg	745 (806)
(lot #101597-134 S.I.= 1.94)	3 µg	13 (8)
	0.3 µg	22 (17)

n = 10 C3H mice per group
i.m. injection day 0, 42; assay d. 63

EXAMPLE 10

Effect of internucleotide linkage on adjuvant properties of ODNs

The results shown in Table 10 demonstrate that certain ODNs with conventional phosphodiester internucleotide linkages can have adjuvant effects when formulated with an HBsAg protein vaccine. Groups of 10 BALB/c mice were co-injected i.m. on days 0 and 21 with HBsAg protein mixed with 30 µg of the indicated ODNs. Control mice were injected with HBsAg protein formulated with or without an adjuvant. Anti-HBsAg titers were determined on sera collected 3 weeks post-dose 2. The results are presented as the GMT (in mIU/mL) with the standard error of the mean in parentheses.

Table 10. Effect of internucleotide linkage on adjuvant properties of ODNs

HBsAg (1 µg) +	Internucleotide Linkage	ODN lot #	Mitogenicity (S.I.)	Anti-HBsAg GMT (mIU/mL)
none	-	-	-	15 (6)
5' GACGTT 3'	phosphoro-thioate	041797-145	23.7	929 (889)
	phospho-diester	041797-143	0.39	189 (140)
5' TCCGGA 3'	phosphoro-thioate	020196-223	0.85	218 (151)
	phospho-diester	020196-224	1.21	286 (150)

EXAMPLE 11

Vaccination of humans

Vaccine formulations containing the 5' GACGTT 3', 5' GAGCTT 3' or 5' TCCGGA 3' adjuvant of this invention can be used to vaccinate humans against a variety of bacterial and viral disease agents. As used herein, human refers to humans of any age that can mount an immune response to an antigen presented by the administration of a vaccine to the human.

In a particular case, a human can be vaccinated against hepatitis B by administration of a vaccine formulation that includes HBsAg and the 5'GACGTT3' adjuvant. The amount of HBsAg in each dose, and the administration schedule, can vary as appropriate for the age of the human. For example, for humans from about birth to about twelve years old, a three dose schedule of from about 2.5 µg to about 5 µg of HBsAg can be administered at 0, 1-3 months afterward and 4-18 months afterward, preferably at 0, 2 and 6 months. The adjuvant in the formulation can be from about 10 µg to about 1,000 - 5,000 µg, and preferably from about 100 µg to about 1,000-2,000 µg per dose or from about 100 to about 500 µg per dose.

For humans between about ten to about twenty years old, a three dose schedule is appropriate. The HBsAg can be present in an amount about 5 µg to about 20 µg, preferably from about 5 µg to about 10 µg per dose, and the 5' GACGTT 3' adjuvant can be present in an amount from about 10 µg to about 2,000-10,000 µg, and preferably from about 100 µg to about 1,000-2,000 µg per dose or from about 100 to about 500 µg per dose. The schedule for administration can be at 0, 2-6 and 4-24 months, preferably at 0, 2-4 and 6-12 months.

For humans from about 18 to about 60 years old, a three dose schedule is also appropriate. The HBsAg can be present in an amount about 5 µg to about 40 µg, preferably from about 10 µg to about 20 µg per dose, and the 5' GACGTT 3' adjuvant can be present in an amount from about 10 µg to about 2,000-10,000 µg, and preferably from about 100 µg to about 1,000-2,500 µg per dose or from about 100 to about 500 µg per dose. The schedule for administration can be at 0, 2-6 and 4-24 months, preferably at 0, 2-4 and 6-12 months.

A therapeutic protocol of treatment for humans is designed for more regular, closely spaced administration of the vaccine. In a therapeutic vaccine, the HBsAg can be present in an amount about 5 µg to about 40 µg, preferably from about 10 µg to about 20 µg per dose, and the 5' GACGTT 3' adjuvant can be present in an amount from about 10 µg to about 2,000-10,000 µg, and preferably from about 50 µg to about 1,000-2,500 µg per dose or from about 100 to about 500 µg per dose. The schedule for administration can be about once every two to six weeks and preferably about once a month for up to about six months or a year.

WHAT IS CLAIMED:

1. An oligonucleotide vaccine adjuvant for the generation of a cell mediated response and an antibody response in an animal to an antigen presented by the administration of a vaccine comprising a hexamer oligonucleotide having a sequence selected from the group consisting of 5'GACGTT3', 5'GAGCTT3' and 5'TCCGGA3'.

2. The vaccine adjuvant of claim 1 wherein at least one of the internucleotide linkages is a non-natural linkage.

3. The vaccine adjuvant of claim 2 wherein at least one of the internucleotide linkages is a phosphorothioate linkage.

4. The vaccine adjuvant of claim 3 wherein all of the internucleotide linkages are phosphorothioate linkages.

5. The vaccine adjuvant of claim 3 wherein at least the terminal internucleotide linkages are phosphorothioate linkages.

6. The vaccine adjuvant of claim 1 wherein said adjuvant is encapsulated in a slow release delivery vehicle.

7. A vaccine composition comprising at least one antigen and a hexamer oligonucleotide having a sequence selected from the group consisting of 5'GACGTT3', 5'GAGCTT3' and 5'TCCGGA3'.

8. The vaccine composition of claim 7 wherein the composition is selected from the group consisting of liquid formulations and lyophilized formulations.

9. The vaccine composition of claim 7 wherein said oligonucleotide is present from about 10 to about 10,000 μg per dose.

10. The vaccine composition of claim 9 wherein said oligonucleotide is present from about 50 to about 5,000 μg per dose.

11. The vaccine composition of claim 9 wherein said oligonucleotide is present from about 100 to about 500 μg per dose.

12. A method of vaccination of a animal comprising:

a) administering a hexamer oligonucleotide of having a sequence selected from the group consisting of 5'GACGTT3', 5'GAGCTT3' and 5'TCCGGA3', and

b) administering at least one antigen.

13. The method of claim 12 wherein said oligonucleotide and said antigen are administered contemporaneously.

14. The method of claim 12 wherein said oligonucleotide and said antigen are administered simultaneously.

15. The method of claim 12 wherein said oligonucleotide and antigen are administered by intramuscular injection at the same site.

16. The method of claim 12 wherein the animal is a human.

17. The method of claim 12 wherein the animal is vaccinated prophylactically.

18. The method of claim 12 wherein the animal is vaccinated therapeutically.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10459

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07H 21/00, 21/04; A61K 48/00
US CL :435/375; 514/44; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/375; 514/44; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS MEDLINE BIOSIS EMBASE CAPLUS SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/02555 A1 (THE UNIVERSITY OF IOWA RESEARCH FOUNDATION) 01 February 1996, see entire document.	1-18
X --- Y --- A	KRIEG et al. CpG Motifs in Bacterial DNA Trigger Direct B-Cell Activation. Nature. 06 April 1995, Vol. 374, pages 546-549, see entire document.	1 ---- 2-6 ---- 7-18
X --- Y	BARR et al. A ^{19}F NMR Study of the Interaction of 3-flouro-4-demethoxydaunomycin with the Hexanucleotide $d(TCCGGA)_2$. Federation of European of European Biochemical Societies. May 1993, Vol. 322, No. 2, pages 173-176, see entire document.	1 --- 2-6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 AUGUST 1998	Date of mailing of the international search report 01 September 1998 (01.09.98)
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SEAN MCGARRY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10459

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids, Linkers and Primers. In: NEW ENGLAND BIOLABS 1993/1994 Catalog, see page 93, see entire document.	1-5